DEVELOPMENT OF NANO FORMULATION COMPOSED OF PHOSPHOLIPID-ENCAPSULATED STANDARDIZED EXTRACT OF *ORTHOSIPHON STAMINEUS* TO ENHANCE THE ANTI-ANGIOGENIC ACTIVITY

By

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This thesis is dedicated to

My wonderful angel, my mother

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
APC	Adenomatous polyposis coli
ATCC	American-type culture collections
BA	Betulinic acid
CO2	Carbon dioxide
ddH2O	Deionised distilled water
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DSC	Differential scanning calorimetry
ECM	Extra-cellular matrix
FAK	Focal-adhesion kinase
FDA	Food and drug administration
FGF	Fibroblast growth factor
h	Hour
HCT 116	Colon cancer cell line
IC50	Inhibitory concentration of 50%
IFN	Interferon
µg/mL	Microgram/millilitre
MHz	Megahertz
min	Minute
mL	Milliliter
mm	Millimeter
mM	Millimolar

MMP	Matrix metallo-proteinase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolum bromide
NaCl	Sodium chloride
N_2	Nitrogen gas
PBS	Phosphate buffer saline
PDGF	Platelet-derived growth factor
PDI	Polydispersity Index
PS	Phospholipids
TNF 1	Tumor necrosis factor 1
RES	Reticuloendothelial system
VEGF	Vascular endothelial growth factor
WHO	World health organization
Wnt	wingless-int , a signal transduction pathway
O.S	*Orthosiphon Stamineus*
EW	Ethanol-water extract
FE	Formulated extract
CAM	Complementary alternative medicine
VHL	Von Hippel-Lindau

LIST OF SYMBOLS

%	Percent
^{o}C	Degree Celsius
α	Alfa
β	Beta
γ	Gama

PENGHASILAN RUMUSAN NANO TERDIRI DARIPADA PHOSPHOLIPID-TERKANDUNG EKSTRAK STANDARD *ORTHOSIPHON STAMINEUS* UNTUK MENINGKATKAN AKTIVITI ANTIANGIOGENIK

ABSTRAK

Orthosiphon stamineus (O.S) telah dibuktikan mempunyai aktiviti antiangiogenik dan antitumor yang berkesan. Namun dos ekstrak yang tinggi diperlukan untuk mencapai kesan klinikal yang efektif. Dalam kajian ini, pendekatan teknologi nano menggunakan pembawa phospholipid telah diterokai untuk meningkatkan aktiviti antiangiogenik ekstrak O.S Hasil kajian ini menunjukkan bahawa nisbah 1: 1 O.S untuk phosphotidylcholine meningkatkan kesan aktiviti antiangiogenik ekstrak O.S formulasi tersebut. Ini menghalang pembentukan tiub endothelium dan penghijrahan sel dan sferoid yang dimansuhkan kanser usus manusia pada potensi yang jauh lebih tinggi daripada ekstrak O.S tidak terformulasi tulen. Saiz zarah penggubalan mempunyai diameter purata 29 nm. Penggubalan tidak bagaimanapun meningkatkan penyerapan asid rosmarinik secara khusus tetapi data HPLC menunjukkan kehadiran rosmarinik kompleks asid-phospholipid. Data juga menunjukkan peningkatan kelarutan sinensitin, eupatorin dan TMF dalam tertib menurun kelarutan. Hasil kajian ini juga menunjukkan bahawa asid rosmarinik bukan komponen aktif utama O.S yang memberikan aktiviti antiangiogenik. O.S-phospholipid kompleks dibentuk menunjukkan potensi yang melebihi asid rosmarinik berkenaan dengan aktiviti antiangiogenik. Kajian ini menyimpulkan satu formula baru yang menjanjikan O.S yang boleh meningkatkan aktiviti antitumor itu.

DEVELOPMENT OF NANO FORMULATION COMPOSED OF PHOSPHOLIPID-ENCAPSULATED STANDARDIZED EXTRACT OF *ORTHOSIPHON STAMINEUS* TO ENHANCE THE ANTI-ANGIOGENIC ACTIVITY

ABSTRACT

Orthosiphon stamineus (O.S) is a promising herb which has been shown to have potent antiangiogenic and antitumor activities. However, a high dose of O.S extract is required in order for it to be clinically effective. In this work, a nanotechnology based approach was adopted to enhance the antiangiogenic activity of O.S extract via phospholipidic carriers. The results of this study showed that a 1:1 ratio of O.S and phosphotidylcholine significantly improved the antiangiogenic activity of the extract. This formulation inhibited endothelial tube formation, and cell migration; and abrogated human colorectal cancer spheroids with significantly higher potency than pure unformulated O.S extract. The formulated particles had an average diameter of 29 nm. Although the formulation did not increase rosmarinic acid absorption specifically, HPLC data indicated the presence of rosmarinic-acid-phospholipids complexes. The data also shows increase in the solubility of sinensitin, eupatorin and 3'-hydroxy- 5, 6, 7, 4'-tetramethoxyflavone in an decreasing order of concentration. Moreover, the results of this study showes that rosmarinic acid was not the principal active component in O.S that conferes the antiangiogenic activity as O.S-phospholipids complexes shows potency which exceeded that of rosmarinic acid alone. The study presented a promising new formulation of O.S that may enhance its antitumor activity.

CHAPTER ONE: INTRODUCTION

1.1. Angiogenesis

1.1.1. Definition, and physiological and pathological mechanisms of angiogenesis

Angiogenesis is the process in which pre-existing vessels develop new blood vessels. It is key to embryology and the development of most tumors (Wu et al., 2002). It also plays a definite role in normal physiological functions, such as reproduction, repair and development (Siddiqui et al., 2009). This process can be regulated by some stimulators like chemokines, growth factors, some special angiogenic enzymes, some endothelial receptors, and adhesion molecules; and also by some inhibitors like angiostatin and endostatin (John et al., 2015). Imbalance between inhibitors and inducers levels can cause a variety of health problems, such as cancer, arthritis, and heart and brain ischemias; and many other diseases (Matsumura et al., 1997). The vascular endothelial growth factor (VEGF) is a growth factor which particularly incites angiogenesis (Ye et al., 1999). As VEGF plays a critical role in some malignant and benign cancers, it can be a suitable target for cancer therapy (Maeda et al., 2009). In order for new blood vessels to be formed, a series of physiological events need to occur. These processes include the dispersion of the extracellular matrix; the migration, adhesion and proliferation of endothelial cells; and tube formation (Ahmadi et al., 1996).

1.1.2. Angiogenesis pathways

Transcription factors like the hypoxia-inducible factors (HIFs) regulate more than 200 genes via hypoxic signaling, hence regulating cell invasion, angiogenesis and mitogenesis (Hawker et al., 2005). Such HIF factors also regulate some proteins, such as Platelet-Derived Growth Factor (PDGF), Vascular Endothelial Growth Factor (VEGF) and Human Enhancer of Filamentation 1. VEGF is expressed in both

primary and metastatic human colon cancers; and its expression is markedly high in metastatic cells (Ahamed et al., 2012). Thus, one of the serum markers used to detect colon cancer in the early stages is VEGF. In hypoxic conditions, the β and α subunits of HIF make dimers and translocate to the nucleus, where they regulate the expression of genes such as those of the vascular endothelial growth factor, Transforming growth factor alpha and Platelet-derived growth factor beta (Ahamed et al., 2012). When the amount of oxygen is normal, prolin and asparagine hydroxylase hydroxylate HIF-1α, setting it to bind to VHL and later be degraded by proteasome (Ahmadi et al., 1996).

1.1.3. Regulation of angiogenesis

Angiogenesis is regulated by a strict balance between mediatory activators and inhibitors. There are three different regulatory stages, which involve the destruction of the extracellular matrix (ECM); adjustments to the levels of angiogenic mediators like cytokines, growth factors and some other enzymes; and interactions on two different levels (cell-cell and cell-matrix interactions), which make for the final stage of regulation of angiogenesis (Gumbiner, 1996).

Matrix metalloproteinases (MMPs) play the main role in the degradation of ECM (Aisha et al., 2014). They break down the protein constituents of ECM and create suitable conditions for cell migration by destroying collagen and other cellular barriers. Hence, MMPs play a key role in tissue turn-over, which resembles a reaction to the environment due to the presence of physiological factors, like the normal growth factors, or pathological conditions, like inflammation and cancer (Aisha et al., 2014). MMPs activity can be regulated by two different ways, either by influencing its expression and activation level by manipulating proteolytic enzymes,

or by adjusting its level of inhibition by manipulating its inhibitors (Al-Suede et al., 2014).

Degradation of ECM due to the effects of different growth factors promotes endothelial cell migration and proliferation, which leads to the formation of new blood vessels (Arnaoutova et al., 2009).

Adhesion to other cells or to their matrices represents another class of regulatory events in angiogenesis, which are strictly regulated in normal cell lines; as errors in the regulation of these events lead to different kinds of human cancers. The process of cell adhesion occurs with the involvement of certain adhesion molecules, ECM components, some endogenous inhibitors and proteolytic enzymes. Unfortunately, decrease in the rate of adhesion promote cancer progression (Carmeliet et al., 2000).

Adhesion molecules are categorized into 5 major protein groups: the selectins, the integrins, the immuneglubin superfamily, the cadherins and the hyaluronans groups. These proteins represent surface receptors which can be targeted to control cancer progression in different cancer types, and offer a variety of therapeutic techniques (Carmeliet et al., 2000).

1.1.5 Tumor angiogenesis

Angiogenesis plays a central role in tumor development. It encompasses a series of interactions between a wide range of intracellular mediators, leading to enhanced endothelial cell proliferation and invasion (Bhattaram et al., 2002).

One of the most important modulators of pathological angiogenesis is VEGF. Loss of one allele of the VEGF gene can illicit major deficiencies in the vascular system, and is lethal in the embryonic stage (Bockhorn et al., 2007).

A certain percentage of somatic mutations can cause normal cells to proliferate continuously, transforming them into cancerous cells (Bravo et al., 2002), whose gradual growth leads to the formation of cancerous tumors. Exaggerated proliferation rates cause cells to need a greater blood supply in order for them to obtain more nutrients. This results in a lack of oxygen and nutrients in normal cells, leading to their death (Chambers et al., 1997). The spreading of tumor cells via the circulatory system, a process called metastasis, enables those cells to reach other organs in the body, causing secondary tumors (Figure 1.1).

When a tumor grows more than its blood supply can allow, HIF-1 is produced, causing VEGF to be transcribed more (Chauhan et al., 2009). VEGF increases the permeability of blood vessels, and causes endothelial cells to migrate and proliferate more than usual. A lack of oxygen can also cause other pro-angiogenic molecules, like nitric oxide synthase and PDGF, to be produced more. It can also affect the levels of growth factors alpha and beta, angiopoietins and the basic fibroblastic growth factors (bFGF) (Chaurasia et al.).

VEGF is one of the most influential molecules which stimulate angiogenesis. Other regulatory molecules include: the basic fibroblast growth factor, the platelet-derived growth factor, and metalloproteinases (MMPs). Moreover, the interferon family (α, β and γ), thrombospondin-1 and -2, angiostatin, and endostatin make a group of endogenous regulators which inhibit angiogenesis (Chen et al., 2009; Chong et al., 2012).

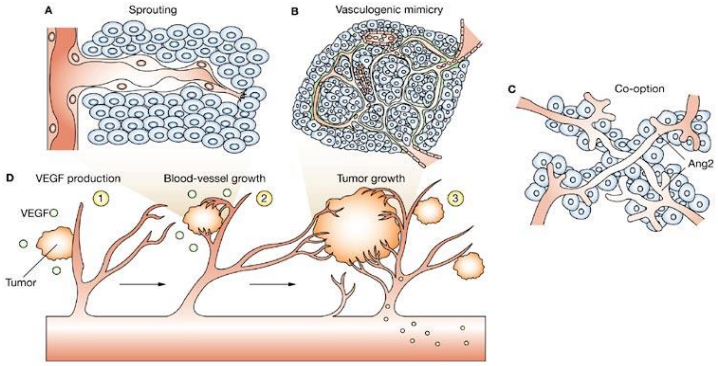


Figure 1.1 Mechanisms of tumor neovascularization (Spannuth et al., 2008).

1.1.4. Mechanisms of angiogenesis therapy

Angiogenesis is an essential process in tumor growth and can be a suitable target for treatment. Hence, the purpose of using anti-angiogenic factors in cancer therapy is to interrupt critical stages of angiogenesis. Anti-angiogenic therapy can lead to reduced vessel permeability and blood perfusion, and vascular shrinkage which decreases the possibility of a tumor receiving oxygen and nutrients (Condeelis et al., 2006). Theoretically, anti-angiogenic therapy may revert tumor blood vessels into the normal state, and improve the quality and delivery of cytotoxic therapeutic agents (D'souza, 2014). Hence, anti-angiogenic agents function by reducing the permeability of blood vessels in tumors, and the dispersion of interstitial fluids. This leads to a decline in interstitial pressure, and, eventually, reduces tumor hypoxia (Maeda et al., 2009). If anti-angiogenic therapy is used along with cytotoxic agents, the effect of these agents increases, while tumor vessels are simultaneously normalized (Damge et al., 1990).

A number of anti-angiogenic therapeutic agents are undergoing clinical trials. These agents can be classified into 3 categories: The first comprises the endothelial

cell growth inhibitors, the preeminent of which is endostatin. This class of angiogenesis inhibitors induces apoptosis and inhibits endothelial cell growth (Decker, 1998). The second category includes drugs which act as angiogenesis-signaling blockers, an example of which is Avastin®. They are inhibitors of the basic fibroblast growth factor (bFGF) and VEGF (Folkman, 2002). The third category is composed of the receptor blockers which inhibit ECM destruction, like the inhibitors of MMPs, which act by inhibiting the receptor activities of multiple growth factors (Guba et al., 2002).

1.2. Medicinal plants as a source of cancer therapeutics

During the previous century, phytochemical research and pharmacological studies have been carried out on numerous herbal extracts to elucidate their chemical compositions and to investigate clinical manifestations associated with the use of herbal remedies. Traditional approaches to treat cancer employ medicines derived from natural sources, such as animals, minerals and herbal products.

Plants can be used as herbal beverage, or can be extracted and their crude extracts may be formulated in the form of capsules or pills (Hawker et al., 2005). Herbal products can be used to manage a variety of health problems, including cancer. There is plenty of evidence indicating that phytochemical products can prevent the progression of cancer (Hood et al., 2002). Phytochemicals can be used by patients who have undergone surgery, those at risk of cancer due to family history, and those undergoing chemotherapy and suffering its side effects (Hou et al., 2012).

1.3 Colorectal carcinoma

According to recent research, the risk factors of colorectal cancer (CRC) include genetic and epigenetic mutations, chronic inflammation, a low fiber diet and an unhealthy lifestyle (Willett et al., 1990). Therefore, preventive measures

implemented to address these issues may help control this malignant disorder. In recent years, a lot of effort has been undertaken exploring new therapeutic compounds derived from natural resources for their potential in preventing and/or treating a variety of cancers, including CRC (Hulkower et al., 2011).

Colon cancer is a multi-stage disease which occurs with the transformation of normal epithelial colon cells into invasive cells (Hüsch et al., 2013).

Many studies have demonstrated that this multi-step process occurs due to mutations in adenomatous polypsis coli (APC) or cyclooxygenase-2 (COX-2) genes; mutations caused by Kirsten-rat sarcoma virus (K-ras mutations); a lack of the 18q21 gene; microsatellites disequilibrium; mutations in transforming growth factor β receptor II (TGFβR2); and/or the translocation and stabilization of the β-catenin gene (Jain, 2003; John et al., 2015; Kieran et al., 2005; Kim et al., 2001; Lamalice et al., 2007; Li et al., 2006).

The effect of the APC suppressor gene in the primary stages of colorectal carcinoma has been well studied. Gene mutations causing a loss of APC function are associated with an accumulation of intracellular β-catenin, which influences cell adhesion, and, like a transcription factor, works on the Wnt signaling pathway (Maeda et al., 2009). Activation of the Wnt/β-catenin signaling pathway results in the activation of T-cell factor/lymphoid enhancing factor-1 (TCF/LEF1), which are transcription factors that promote the expression of specific target genes, like c-Myc, cyclin D1 and COX-2, which are responsible for the tumorigenesis of colorectal carcinoma and a few other cancers (Mannello et al., 2001). COX-1 and COX-2 are different isoforms of cyclooxygenase (COX). Studies have shown that over expression of COX-2 leads to human colon cancer and adenomas. As the conversion of arachidonic acid to prostaglandins is mediated by COX-2, higher

levels of this enzyme may stimulate the proliferation of cancer cells, stimulate angiogenesis, and/or inhibit apoptosis. This is because prostaglandins promote the production of several growth factors, like VEGF and hepatocyte growth factor, which stimulate tumor angiogenesis and the proliferation of cancer cells (Matsumura et al., 1997).

Other types of genetic disorders involving unstable microsatellites and chromosomes can also lead to carcinogenesis in the colon. Chromosomal instability results from a series of genetic alterations, mainly involving the stimulation of oncogenes like K-ras; the inactivation of tumor-suppressor genes, such as TP53 and APC; and the deletion of the colorectal carcinoma gene (DCC), which is located on chromosome 18q21. Mutations of the sort are associated with an increased risk of relapse and death among colon cancer patients (Misra et al., 2003). On the other hand, a deficient DNA mismatch-repair mechanism can lead to unstable microsatellites, and can increase the frequency of mutations, especially in the repetitive microsatellite sequences (Murugan et al., 2009).

Colorectal cancer is highly angiogenesis-dependent; and it ranks third in terms of worldwide cancer incidence, preceded only by lung and breast cancers. It accounts for almost 10% of total cancer cases, and almost 8% of total cancer deaths worldwide (Murugan et al., 2009). Unfortunately, according to the World Health Organization (WHO), more than 70% of all cancer deaths occur in countries with low or middle income; and cancer-related deaths are projected to continue rising worldwide to reach over 11 million in 2030 (Picker et al., 2014).

1.4 Colon cancer chemotherapy strategies

There is no specific treatment for any of the different types of cancer to date. Hence, the primary aim of cancer treatment is prolonging the patient’s life

expectancy while ensuring a good quality of life. The principal chemotherapeutic agents employed in the treatment of colorectal carcinoma include cytotoxic agents, such as those consisting of 5-fluorouracil (5-FU), irinotecan, oxaliplatin (OX), leucovorin (LV) and capecitabine (Cap). Drugs representing different combinations of these agents, such as IFL (irinotecan, 5-FU and LV), FOLFOX (5-FU, OX and LV), FOLFIRI (5-FU, LV and irinotecan) and CapOx (capecitabine/oxaliplatin), have been studied in phase II and phase III clinical trials; and their therapeutic efficacies make them superior to every one of these agents alone (Sahu et al., 2015).

Moreover, numerous combinations of monoclonal antibody agents, such as bevacizumab (anti-VEGF), panitumumab (human anti-EGFR) and Cetuximab (chimeric human-mouse anti-EGFR), and cytotoxic drugs have been studied in phase II and phase III clinical trials.

In general, the results indicated that combinations of either anti-VEGF or anti-EGFR antibodies and a cytotoxic agent offer a better therapeutic efficacy than each one of their components individually (Sahu et al., 2015).

However, some combinations, like those involving anti-VEGF and anti-EGFR with irinotecan, have been shown to cause a negative effect on the therapeutic outcome and the molecular status of tumors, reducing the availability of the wild-type or increasing the concentration of mutant K-ras (Sahu et al., 2015).

1.5 Herbal products as cancer treatments

Natural drugs have been used for decades to cure several diseases. Like many kinds of supplementary or, sometimes, alternative treatments, most people on earth have for long used herbs to help themselves feel better or to gain more control of medical conditions like hay fever, painful bowel syndrome, menstrual (period) difficulties and skin problems such as eczema (Caudill, 2008; Saraf,

2010). A number of studies estimate that approximately 6 out of each 10 people with cancer (60%) use herbal products combined with conventional cancer therapeutic agents (Richardson et al., 2000). There are many types of therapeutic herbal products, several of which overlap with food items. Plants like echinacea, St John's wort, green tea and ginger (Shelke, 2012) are frequently used as remedies.

There is an increasing trend worldwide for the utilization of supplementary natural products and complementary alternative medicine (CAM) to aid or substitute conventional drugs in the management of various health problems, like the generalized body weakness resulting from continuous use of standard chemotherapeutic agents (Poonthananiwatkul et al., 2015). Phytochemical products from plant roots, bulbs, barks, leaves, and stems as well as other herbal components have demonstrated potential beneficial qualities, like anti-cancer activities. They have even been shown to provide ingredients useful for the synthesis of modern drugs (Shojaei et al., 2007). A number of studies on herbal drugs have resulted in the identification of compounds with promising biological activities. Thus, investigations directed towards the determination of the proper dosage regimens of these herbs/compounds are highly sought to improve the treatment outcomes.

1.6 Bioavailibility of phytonutrients

Studies have shown that many naturaceutical agents, including those derived from natural products, demonstrate poor bioavailability when consumed orally (Kuhrts, 2012). For instance, the oral bioavailabilities of many compounds from *Eurycoma longifolia* J. (Tongkat Ali), *Andrographis paniculate* (Hempedu Bumi),

and *Orthosiphon stamineus* (Misai Kucing) have been found to be less than 1% (Bhattaram et al., 2002).

The major bioactive compounds in *O. stamineus* are polyphenols and flavonoids (e.g. rosmarinic acid, 3',4',5,7-tetramethoxyflavone, etc.) (Siddiqui et al., 2009). It was reported that the poor bioavailability of flavonoids was due to their large particle sizes and their poor miscibility with oils and other lipids, which is ought to hinder the absorption of flavonoids by the membranes of the small intestines (D. Singh et al., 2011; Staton et al., 2004). Lipid solubility and particle size are the major factors preventing certain molecules from passing through biological membranes and being systematically absorbed following oral administration (Tripathi, 2013). The effectiveness of an herbal product or medication is dependent upon delivering an effective level of its active compounds to the blood stream (Staton et al., 2004).

Most of the bioactive phytoconstituents, like flavonoids, terpenoids, tannins, xanthones, polyphenols, etc. are water souble, (A. Singh et al., 2011) and, hence, have bad absorption prfiles (Suryawanshi, 2011).

1.6.1 Nanotechnology in drug delivery systems

So far, most of the new potential therapeutics have demonstrated poor pharmacokinetics and biopharmaceutical properties. Therefore, enhanced drug delivery systems are warranted to allow the distribution of therapeutically active drug molecules to the site of action, while minimizing the exposure of healthy organs and tissues to these molecules as much as possible. Nanotechnology may serve this purpose by playing a key role in the development of future therapeutic agent "nanomedicines". These new therapeutic agents are sought to necessitate lower doses

and offer better therapeutic effectiveness and safety profiles. To be considered efficient delivery systems, nanoparticles are supposed to be in the nanometer-size range, preferably between 1 and 200 nm, and must be able to contain encapsulated, dispersed, adsorbed, or conjugated drugs and imaging agents (Yen et al., 2009).

1.6.2 Methods to improve bioavailability

Low bioavailability is the major shortcoming in terms of the therapeutic potential of plant-based drugs. To address this issue the development of good formulations is required to improve the bioavailability and enhance the therapeutic effectiveness of such drugs. A liposome is produced by the addition of a water soluble compound to phosphatidylcholine (Tung et al., 2011). No chemical bonds are created, but it is rather that the molecules of phosphatidylcholine act as a group to enclose the water-soluble compound. Lots of phosphatidylcholine molecules are needed to surround a compound fully.Phytophospholipid conjugated herbal extracts (phytosome extracts) are prepared by mixing water-soluble herbal extracts with phosphatidylcholine, which results in the generation of chemical bonds between certain plant constituents and phosphatidylcholine. 1:1 or 2:1 stoichiometric complexes are usually what makes up phytosomes. They result from interactions between an extract or a phytoconstituent and a phospholipidic molecule, leading to enhanced absorption of bioactive constituents as compared with liposomes in which no interactions takes place between the herbal extract and phosphatydilcholine (Valster et al., 2005). Compared to phytosomes, among the few drawbacks of liposomes is that they are more deficult to formulate, pose higher production costs, and have a greater tendency to leak the encapsulated drugs. Free phospholipids are sometimes prone to oxidation, hydrolysis and other reactions, causing them to have short half lives, poor solubility, stability issues, immunogenic effects, and limited

target availability. They may also be rapidly cleared from the circulation as a result of their uptake by the reticuloendothelial system (RES), which primarily exists in the liver (Wu et al., 2002). On the other hand, formulations involving phytosomes have been shown to be safe; and their components have all been approved for pharmaceutical use (Di Pierro et al., 2013).

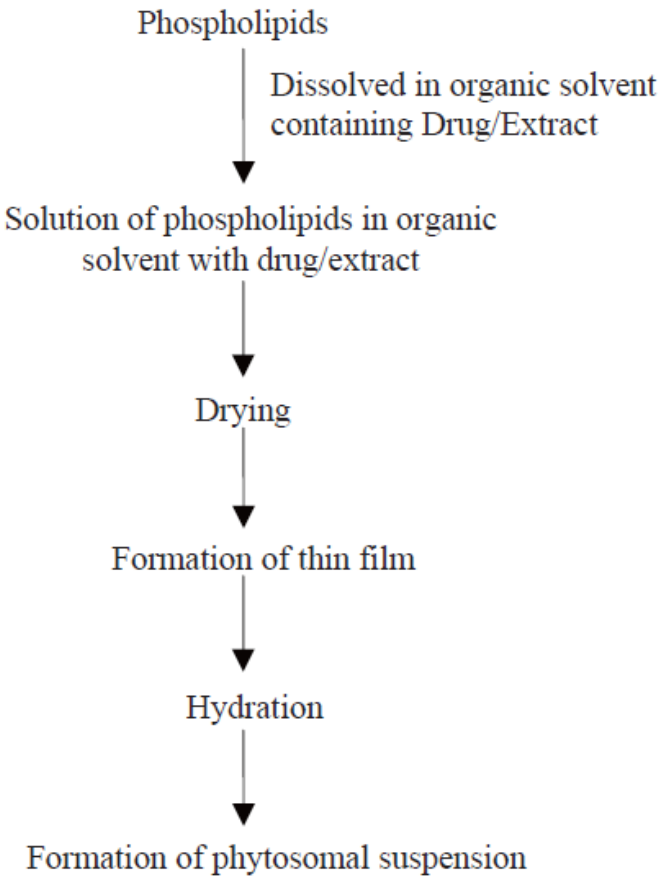


Figure 0.1: Phytosome preparation method (Patel et al., 2009).

1.6.3 Phytophospholipid complexes as drug delivery systems

Phospholipids are useful carrier molecules utilized as a drug delivery technology. They are essential vehicles for the drug molecules which need to be administered in controlled release forms (Xu et al., 2013). The preparation of drugs in lipid complexes may lead to improved solubility and decreased GI toxicity (Yamaguchi et al., 2007). Such amphiphilic drug–lipid complexes are stable and

possess good bioavailability, which is why they are widely used in today´s drug delivery systems (Yamazaki et al., 2005). Decreased interfacial tension between a delivery system and GI fluids facilitates drug movement through the membranes, tissues, and cell walls in the organism (Ye et al., 1999).

Phytosomes are phospholipid complexes of drugs possessing amphiphilic properties and hydrogen atoms capable of binding to phospholipids (Zhang et al., 2013). Phytosomes impart better pharmaceutical and biological properties than the drug alone, leading to increased bioavailability (Saraf, 2010). Those complexes have been prepared for different non-steroidal anti-inflammatory drugs, antineoplastic drugs and some proteins. Formulating a drug as a phytosome complex increases its absorption, and minimizes its gastrointestinal toxicity (Carmeliet, 2000).

However, these novel carriers should have two important properties: First of all, they should deliver the drug at a specified rate subject to the patient's body needs during treatment. Second, they should direct the active compounds in herbal drugs to the targeted site of action. Standard dosage forms allowing prolonged-release of a drug do not normally possess such properties.

In phyto-formulation research, nano dosage forms, such as polymeric nanoparticles, nanocapsules, solid-lipid nanoparticles, nanoemulsions and phytosomes, offer some advantages, like enhanced solubility and bioavailability, improved pharmacological activities, and decreased toxicity. Hence, research is currently focused on finding ways to protect phytosomes from degradation and achieve better stability profiles. Herbal nano drug delivery systems are expected to overcome most of the problems related to herbal medicine use in the future (Saraf, 2010), as phytosomes, which are made by binding particular components of herbal extracts with phosphatidylcholine, represent a dosage form with superior absorption

rates and better levels of effectivity compared to unformulated traditional herbal extracts.

1.7 Phytophospholipid complexes overview

1.7.1 Definition of a phytophospholipid complex

The Greek word “phyto” signifies a plant; and “some” is often used to mean cell-like. Hence, a “phytosome” is an herbal cell-like construction. It refers to an advanced natural formula consisting of bioactive constituents of plant extracts encapsulated in lipid (Carmeliet et al., 2000).

1.7.2 Rationale of phytosome formulations

Many of the bioactive components of phytomedicines are water-soluble in nature. This includes flavonoids, glycosides, tannins and terpenoids. As flavonoids make for a large category of bioactive compounds, they have been shown to possess a wide range of curative activities (Carmeliet et al., 2000; Chaurasia et al.). However, they are improperly absorbed. This is in part due to their large molecular sizes, which cannot really allow them to be absorbed by passive diffusion. It is also possibly due to their weak lipid solubility, which significantly restricts their capability to go across the lipid-rich biological membranes in the human body.

Phytosomes offer superior pharmacokinetic and pharmacodynamic profiles compared with traditional natural extracts. Phytosome technological innovations have been appropriately employed to promote the bioavailability of several therapeutic herbal extracts, including the extracts of milk thistle, *ginkgo biloba*, grape seeds, green tea, hawthorn, and ginseng, allowing them to be marketed for different medicinal applications and as nutritional supplements (Harris et al., 2010). Phytosomes can also be possibly utilized in pharmaceutical preparations to achieve

better anti-inflammatory actions and allow phytosome extract cosmetic combinations.

1.8 Phytosome technology

A phytosome forms as a micro sphere structure around a plant extract and its active components, and provides a protective layer against gastric secretions and gut bacteria alike (Patel et al., 2009). Phytosomes are obtained via a reaction between soy phospholipids and particular botanical derivatives in an appropriate solvent. On account of their physiochemical and spectroscopic characteristics, some complexes of the sort might possibly be considered novel systems of drug delivery (Koo et al., 2005).

1.9 Characteristics of a phytosome

1.9.1 Chemical characteristics

A phytosome is a complex formed between an herbal product and a natural phospholipid, such as soy phospholipids. Basically, the choline heads of the phosphatidylcholine molecules bind to the herbal constituents; and then, the lipid-soluble phosphatidyl portions, which compose the body as well as the tails of these molecules, envelope the choline-bound components (Shelke, 2012). However, there is complexity regarding the outcome of adding different stoichiometric quantities of a phospholipidic material to a chosen polyphenolic compound, which might be a simple flavonoid, in a non-polar solvent (Spannuth et al., 2008).

1.9.2 Biological properties

Pharmacokinetic and pharmacodynamic analyses in experimental animals and human subjects have been made to reveal the natural tendencies of phytosomes (Harris et al., 2010). The enhanced bioavailability of phytosomes in contrast with

non-complexed botanical derivatives continues to be the focus of researches worldwide (Caudill, 2008).

1.10 Advantages of phytosomes over conventional dosage forms

Great improvement in the bioavailability of botanical extracts can occur upon being formed into complexes with phospholipids. This process yields better absorption in the intestinal tract. It allows non-lipophillic botanical extracts to readily permeate the intestinal lumen, something which is, in any other case, impossible (Saraf, 2010).

Phosphatidylcholine, an extremely important part of a cell's membrane, has been utilized in phytosome technology to act as a carrier, and to aid in skin nourishment (Valster et al., 2005).

Phytosomes are more stable than liposomes as chemical bonds are formed not only between the phosphatidylcholine molecules themselves but also between them and the phytoconstituents present (Richardson et al., 2000). Phytosomes are not to be mistaken for liposomes. They are structurally quite different. As opposed to a phytosome, a liposome is formed by combining a water-soluble compound with phosphatidylcholine. Not a single chemical covalent bond is created. It is rather that the phosphatidylcholine molecules enclose the water-soluble compound (Poonthananiwatkul et al., 2015). A great number of phosphatidylcholine molecules might be necessary to enclosing a water-soluble product. Conversely, with the application of a phytosome procedure, phosphatidylcholine molecules and a particular herbal extract basically form 1:1, or perhaps 2:1, complexes, based on the contents of the extract. Medicinal liposomal complexes are produced in the presence of water or a buffer solution. This allows the phytosomes to react with the solvent and obtain lower dielectric potentials (Carmeliet et al., 2000). With regards to

phytosome supplements, many studies have shown that they can be significantly better absorbed and present significantly enhanced biological effectiveness (Saraf, 2010).

1.11 Phytophospholipid complexes in cancer chemotherapy

Numerous dietetic vegetables, therapeutic herbs and other plants have been studied and their cancer chemopreventative efficacies evaluated. Consumption of herbal supplements may have a considerable therapeutic value in terms of reducing the risk of cancer and the side effects of chemotherapy (Kuhrts, 2012). Herbal compounds can aid in reducing the inflammatory episodes which play a critical role in carcinogenesis (Tripathi, 2013). It has been estimated that almost one-third of all cancer-related deaths in America could possibly be avoided by better dietetic choices (A. Singh et al., 2011). Emerging data implies that numerous dietetic agents and medicinal herbs can be utilized solely or in combinations alongside conventional chemotherapeutic agents to halt cancer progress; avoid the adverse incidence of cancer and metastasis; and, possibly, cure cancer (Mosmann, 1983; Yen et al., 2009).

1.12 Traditional uses of *O. stamineus*

Orthosiphon stamineus Benth. (Lambiaceae) is an important medicinal herb found largely in South East Asia, where its areal parts have been traditionally used as remedy. Various *in vitro* and *in vivo* models have been established and utilized to study the bioactive phytochemicals, including the flavonoids, terpenoids and essential oils, present in this herb (Belcaro et al., 2013). Early traditional practitioners used the leaves of *O. stamineus* (O.S) as a diuretic, and incorporated them into preparations intended for the removal of kidney stones. However, later in time, the leaves started to be utilized to prevent and cure gallstones; rheumatism;

diabetes mellitus; hypertension; tonsillitis; epilepsy; menstrual disorders; gonorrhea; syphilis; acute and chronic nephritis; gout; osteoarthritis; and psoriasis (Ribatti, 2011). Previous reports have demonstrated *O. stamineus* versatile pharmacological activities, which are attributable to the presence of a number of bioactive phytochemicals in this herb. *O. stamineus* possesses broad pharmacological properties which can be beneficial in different pathophysiological conditions. Thus, O.S can be considered a good candidate for further experimental and medical research (Willett et al., 1990).

1.12.1 Pharmacological properties of *O. stamineus*

There has been much research work on O.S which have revealed quite a number of interesting and important pharmacological properties of this plant. The pharmacological effects of O.S are attributable to the existence of polyphenols, glycosides, lipophilic flavones, triterpenes, and diterpenes in its extracts, presenting a number of bioactive compounds, such as rosmarinic acid (RA), sinensetin (SIN), eupatorin (EUP), betulinic acid, olenolic acid, 3′-hydroxy-5, 6, 7, 4′-tetramethoxyflavone (TMF) and several caffeic acid derivatives (Di Pierro et al., 2013) which have been considered to be key chemical markers that gave the biological activities observed (Di Pierro et al., 2013).

A phytochemical analysis of O.S has shown a large number of chemical components, including monoterpenes, diterpenes, triterpenes, saponins, flavonoids, essential oils and several long-chain organic acids, which still need to be explored and identified. Pharmacological evaluation of different O.S extracts, tinctures, fractions, and even pure isolated compounds have shown to possess antioxidant, antimicrobial, anti-angiogenic, antitumor, diuretic, nephroprotective, antidiabetic,

antihypertensive, anti-inflammatory, anti-obesity and hepatoprotective activities (Yuliana et al., 2011).

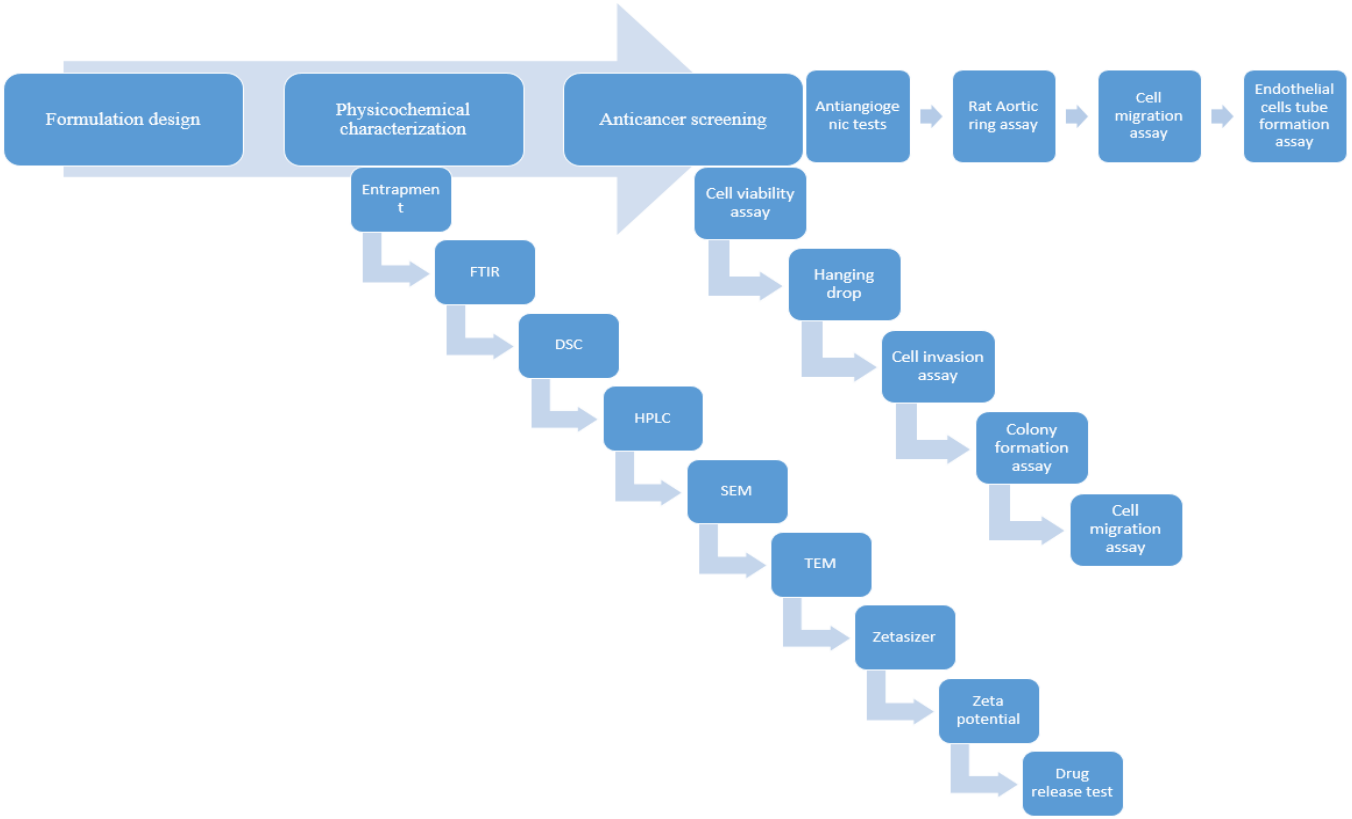
1.13 Aims and objectives

Previously it was shown that ethanol extract of O.S can cause significant inhibition of angiogenesis (Ahamed et al., 2012). The studies showed that the extract can inhibit colon cancer in vivo and targets VEGF expression and inhibits the phosphorylation of the VEGFR receptor (Ahamed et al., 2012). The activity was found to be dose dependent and rosmarinic acid was hypothesized to be the main active compound given its large amount in the extract. Other compounds present in the extract includes eupatorin, sinsnsetin and TMF. All these compounds as mentioned previously are established anti-oxidants and their bioavailability are limited (Akowuah et al., 2005). Thus we hypothesize that encapsulating the O.S extract with phospholipid may help to improve the bioavailability of the phenolic and flavonoids of the O.S extract and consequently can help to enhance the pharmacological effect, in particular antiangiogenic activity. Hence to validate this hypothesis, the following general objectives are being set:

1. To develop a nano-formulation of *O. stamineus* extract and elucidate its physico-chemical properties.
2. To enhance the antiangiogenic effect of the nano-formulation of *O. stamineus* to exceed that of *O. stamineus* crude extract.
3. To develop a drug delivery system for *O. stamineus* nano-formulation to achieve maximum bioavailability.

CHAPTER TWO: MATERIAL AND METHODS

Flowchart of the research



Materials and instruments

2.1.1. Materials

Acetone	Sigma-Aldrich, USA
Agar	Fisher, USA
Agarose	Fisher, USA
Aluminium chloride	Sigma-Aldrich, USA
Amphotericin B (Fungizon)	Sigma-Aldrich, USA
Aprotinin	Sigma-Aldrich, USA
Betulinic acid	Sigma-Aldrich, USA
CCD-18Co	ATCC, Rockville, MD, USA
Chloroform	Riedel-de Haën, Germany
Crystal violet	Sigma-Aldrich, USA
DMSO	Sigma-Aldrich, USA
DMEM medium	Gibco/Life technology, UK
ε-aminocaproic acid	Sigma-Aldrich, USA
EA.hy926	ATCC, Rockville, MD, USA
ECGS	ScienCell, USA